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(54) Title: BLUEBERRY RED RINGSPOT VIRUS, SEQUENCES, PROMOTERS, AND USES THEREOF

(57) Abstract: A nucleic acid sequence of the blueberry red ringspot virus is disclosed. Also disclosed are putative promoter regions of the sequence and promoter regions capable of directing transgene expression in plants, including tissue-specific expression. Also disclosed are expression vectors, transformed plant cells and plants containing a blueberry red ringspot virus promoter and an encoded product for expression. Methods for diagnosis of blueberry red ringspot virus infection are also provided.

BLUEBERRY RED RINGSPOT VIRUS, SEQUENCES, PROMOTERS, AND USES THEREOF

RELATED APPLICATION

The present application claims the benefit of U.S. provisional application Ser. No. 60/318,050 filed Sep. 7, 2001, which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

(1) Field Of The Invention

This invention relates to nucleic acid sequences of the blueberry red ringspot virus and, more particularly, to the nucleic acid sequence of the virus, the identification of promoters, and the use of the promoters in the expression of recombinant genes in transgenic plants, including tissue-specific expression in plants. The invention also relates to sequences of the blueberry red ringspot virus useful in the diagnosis of disease in plants, and related methods thereof.

(2) Description Of The Related Art

Recombinant viral promoters can be used to direct the expression of operably linked heterologous genes. Such expression can occur in a transgenic environment. For expression of transgenes in plants, a promoter from Cauliflower Mosaic Virus (CaMV) is widely used, for example as disclosed in US Patent 6,255,560 to Fraley et al. Expression of transgenes in plants under the control of a CaMV promoter tends to be at high levels and show little tissue- or cell-type specificity. This virus is unusual, in that it appears to comprise only two promoters. One promoter appears to control the transcription of the entire viral genome into an RNA copy. This promoter (the "35S" promoter) is a tandem repeat of an approximately 350 base pair sequence. Within this sequence are domains involved with tissue specific expression of genes expressed from the promoter (Odell, J.T., Nagy, F. and Chua, N.H. (1985) Identification of DNA sequences required for the activity of the cauliflower mosaic virus 35S promoter. *Nature* 313: 810-812; Benfey, P.N. and Chua, N.H. (1990) The Cauliflower Mosaic Virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 250: 959-966; Daubert, S.D., et al., (1984) Expression of Disease Symptoms in CaMV Genomic Hybrids. *Journal of Molecular and Applied Genetics* 202: 1043-1045; Dixon, L.K., et al., (1983) Mutagenesis of Cauliflower Mosaic Virus. *Gene* 25: 189-199; Mesnard, J., et al. (1990) The Cauliflower Mosaic Virus Gene III

product is a non-sequence specific DNA binding protein. *Virology* 174: 622-624; Pfeiffer, P. and Hohn, T. (1983) Involvement of reverse transcriptase in the replication of cauliflower mosaic virus. *Cell* 33: 781-789; Takatsuji, H., et al. (1992) Cauliflower Mosaic Virus reverse transcriptase — activation by proteolytic processing and functional alteration by terminal deletion. *Journal of Biological Chemistry* 267: 11579-11585; Thomas, C. L. et al. (1992) A mutation in Cauliflower Mosaic Virus Gene I interferes with virus movement but not with virus replication. *Virology* 74: 1141-1148). This promoter has proven useful for directing the expression of heterologous genes in transgenic plants.

Because transgenes under CaMV promoter control may not be suitable for all uses, there is a need for the identification and characterization of more plant virus promoters. For example, there is the need for tissue specific promoters that can direct expression of an operably linked gene to a subset of tissues within a transgenic plant. Furthermore, there is a need for a strategy for identifying putative promoters, and a further need to demonstrate the operability of a putative promoter in a transgenic environment.

The blueberry red ringspot virus is a virus with a limited host range: it is believed to infect only blueberry plants. Disease symptoms are observed primarily in the months of July, August and September, and comprise red spots primarily on upper leaf surfaces (Hutchinson, M.T. (1950) Can you recognize the symptoms of stunt disease? *Proceedings 19th Annual Blueberry Open House* 19: 9-11; Ramsdell, D.C., Kim, K.S. and Fulton, J.P. (1987) Red Ringspot of Blueberry. In: Converse, R.H. (ed.) *Virus Diseases of Small Fruits.* U.S. Department of Agriculture, Agr. Res. Svc., Washington, DC Handbook 631; Kim, K.S., Ramsdell, D.C., Gillett, J.M. and Fulton, J.P. (1981) Virions and substructural changes associated with blueberry red ringspot disease. *Phytopathology* 71: 673-678; Gillett, J.M. (1988) Physical and Chemical properties of Blueberry Red Ringspot Virus. Master's thesis, Michigan State University; Hutchinson, M.T. and Varney, E.H. (1954) Ringspot: A virus disease of cultivated blueberry. *Plant Disease Reports* 38: 260-262). A sequence of a putative blueberry red ringspot virus has been published on the World Wide Web at http://www.ncbi.nlm.nih.gov/ with accession numbers NC 003138 and AF404509. This sequence has a length of 8,303 base pairs. No promoters are disclosed or identified in these sequence listings. Furthermore, this sequence has several differences with the sequence of the virus of the present invention as disclosed herein.

BRIEF DESCRIPTION OF THE INVENTION

The present invention involves the discovery and characterization of the nucleic acid sequence of the genome of blueberry red ringspot virus (BRRV). The genome comprises a circular, double-stranded DNA molecule. The molecule has a length of at least 8,241 base pairs. Promoters are identified in the sequence based upon analysis of sequence structure. Recombinant promoters derived from BRRV DNA are used to direct gene expression.

In some embodiments, the present invention is directed to a fragment of the viral nucleic acid which putatively functions as a promoter in plant cells. Each of these putative promoter regions comprises a "TATATAA box" or a "TATA box" and a nearby open reading frame located 3' to the TATATAA box or TATA box. In another embodiment, a consensus sequence for the blueberry red ringspot putative promoters is provided. Also within the scope of the invention are methods for identifying putative promoter sequences of the Blueberry Red Ringspot Virus.

In preferred embodiments, the invention is drawn to a recombinant nucleic acid comprising a fragment of the full-length nucleic acid of the BRRV which functions as a promoter (a "BRRV promoter"). In a preferred embodiment, the present invention provides a recombinant nucleic acid comprising a BRRV promoter operably linked to a gene for expression as a transgene in a host organism or cell. The transgene preferably encodes a polypeptide or a regulatory RNA molecule. Preferably, the host organism is a plant, and the host cell is a plant cell. In another preferred embodiment, a recombinant nucleic acid comprising a BRRV promoter operably linked to a gene for expression as a transgene, provides tissue-specific expression of the transgene in a transformed host plant or a descendant thereof. Preferably, the tissue-specific expression is directed to root tissue or to leaf tissue. More preferably, the tissue-specific expression is directed to root tissue.

In some embodiments, the invention is drawn to a transgenic plant or a transgenic plant cell comprising a recombinant BRRV promoter operably linked to a transgene. In some embodiments, promoter modifications, for example deletions and tandem duplications, provide alternative promoter constructs providing altered transcription patterns. Altered transcription patterns comprise alterations in quantity, timing, and/or tissue specificity in the expression of an operably linked transgene.

The present invention is directed, in some embodiments, to the nucleic acid sequence of the blueberry red ringspot virus and to fragments thereof comprising at least 10 consecutive nucleotides of the full-length sequence. Such fragments

provide probes and primers to detect blueberry red ringspot virus DNA in diagnostic tests to ascertain whether a susceptible host plant is infected with the virus.

In some embodiments, the present invention is directed to an isolated nucleic acid molecule comprising a nucleotide sequence capable of hybridizing under stringent conditions to a nucleic acid comprising a sequence of the blueberry red ringspot virus, or a complement thereof.

Among the several advantages achieved by the present invention, therefore, may be noted: the nucleic acid sequence of the blueberry red ringspot virus genome; putative promoters of the blueberry red ringspot virus; a consensus sequence of blueberry red ringspot virus promoters; a vector comprising a recombinant BRRV promoter operably linked to a sequence encoding a polypeptide or an RNA; a transgenic plant or transgenic plant cell comprising a recombinant BRRV promoter operably linked to a sequence encoding a polypeptide or an RNA; a recombinant BRRV promoter that can direct expression of an operably linked DNA sequence encoding a polypeptide or an RNA; a recombinant BRRV promoter that can direct tissue-specific expression of an operably linked DNA sequence encoding a polypeptide or an RNA; a method for transforming a plant or a plant cell with a DNA molecule comprising a recombinant BRRV promoter and an operably linked DNA sequence encoding a polypeptide or an RNA; and a diagnostic method for detecting the presence of blueberry red ringspot virus in a host plant.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 represents a map (not drawn to scale) of the blueberry red ringspot virus genome.

FIG. 2 represents a comparative alignment of blueberry red ringspot virus putative promoter sequences and a consensus DNA sequence for a putative blueberry red ringspot virus promoter derived from the sequence data (SEQ ID NO: 10).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, blueberry red ringspot virus, the pathogen responsible for causing red ringspot disease in blueberry plants, has been sequenced and characterized by standard methods, such as those disclosed in Sambrook, J. C., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

The virus comprises a circular genome of double stranded DNA. A linearized map of the circular genome (and its putative promoters and open reading frames, discussed below) is shown in figure 1, and the sequence in linearized form (SEQ ID NO: 1) is shown in figure 2. The virus is sequenced by subcloning restriction fragments into sequencing vectors. Fragments are generated using more than one restriction enzyme, and common sequences are used to order the sequence fragments. Because isolates of the virus from different individual infected plants provide slightly different sequences, a full-length sequence of a BRRV DNA can be different from the sequence disclosed herein. The BRRV sequence comprises at least 8214 base pairs. The nucleic acid sequence of the virus in the present invention (SEQ ID NO: 1) represents a composite assembled from sequences from several isolates and comprises most of the BRRV sequence.

Fragments of the full-length sequence are used as probes and/or as PCR primers to detect the presence of the virus in susceptible host plants according to methods know in the art. Such fragments can be, preferably, at least 10 consecutive nucleotides in length, at least 15 consecutive nucleotides in length, at least 20 consecutive nucleotides in length, at least 50 consecutive nucleotides in length, at least 100 consecutive nucleotides in length or greater up to the full-length viral sequence. Such fragments or complements thereof specifically hybridize to the blueberry red ringspot DNA sequence as shown in Figure 2, or the complement thereof. Specific hybridization results in hybridization of the fragments or complements thereof to the blueberry red ringspot virus or to a target sequence thereof, preferentially over other DNA molecules that might be present in a sample tested. Hybridization conditions for obtaining such specificity, preferably, involve high stringency conditions as are well know in the art (see Sambrook et al, *supra*).

Diagnostic methods based upon the probes and/or primers are also within the scope of the present invention. In one diagnostic approach, a nucleic acid sample obtained from a plant suspected of containing the virus is incubated with a nucleic acid probe of the present invention. Incubation conditions are such that the probe will specifically hybridize with any blueberry red ringspot virus nucleic acid that might be present in the sample. Any resultant hybridization complexes formed are then detected using methods well known in the art.

PCR methods are also contemplated in diagnostic methods in which, preferably, two oligonucleotide primers of the present invention serve as a primers for a polymerase chain reaction method. Primers are selected from the sequence as

shown in Figure 2 or the complement thereof, to flank a target sequence which lies within a blueberry red ringspot virus nucleic acid. Any target DNA that might be in a nucleic acid sample obtained from a plant suspected of containing the virus is then amplified by standard methods using the primers. Criteria for selecting primer sequences are well known in the art. The presence of an amplified target DNA sequence is then detected by methods known in the art.

In some embodiments, the present invention includes methods of identifying putative promoters of the blueberry red ringspot virus. In this method, BRRV sequences with the properties of "TATATAA boxes" are identified using computer and/or manual methods (see, e.g., Boyer, T.G., and Maquat, L.E. (1990). Minimal sequence and factor requirements for the initiation of transcription from an atypical TATATAA box-containing housekeeping promoter. *J. Biol. Chem.* 265: 20524-20532). In preferred embodiments, sequence analysis is conducted with the aid of a digital computer programmed with algorithms known in the art, preferably a GCG (University of Wisconsin-Madison) "FIND" software program. In this method, TATATAA-box elements within the BRRV sequence are examined for ATG translational start sites and for classical cis-acting elements, such as AS-1/AS-2-like motifs and GATA boxes. The presence in a sequence of at least one TATATAA-box, at least one classical cis-acting element, and at least one translation start site located 100 base pairs or less downstream from the TATATAA-box provide criteria for designating a BRRV sequence as a putative BRRV promoter.

Analysis of the sequence of the blueberry red ringspot virus by the above method reveals the presence of putative promoters and open reading frames. Seven putative promoters identified using the above criteria are designated "BRRV Promoter A" (SEQ ID NO: 2), "BRRV Promoter B" (SEQ ID NO: 3), "BRRV Promoter C", (SEQ ID NO: 4) "BRRV Promoter D" (SEQ ID NO: 5), "BRRV Promoter E" (SEQ ID NO: 6), "BRRV Promoter F" (SEQ ID NO: 7), and "BRRV Promoter G. (SEQ ID NO: 8)" Their positions in the blueberry red ringspot virus genome are determined by referring to the map in figure 1 or the BRRV sequence (SEQ ID NO: 1). In addition, figure 1 indicates the relative positions of known open reading frames with respect to the putative promoters. The presence of 7 putative promoters is an unexpected result; another virus within the same family (*Caulimoviridae*), the cauliflower mosaic virus, is known to comprise only 2 promoters, including at least one which transcribes the entire genome. It is believed that the promoters of the present invention can function as monocotyledonous and dicotyledonous plant cell promoters. However,

functional assays of recombinant putative promoter (see below) have revealed promoter activity from only two BRRV promoters.

Open reading frame (ORF) regions are identified as the portion of the sequence extending after the TATA-box element from a start codon to a stop codon. ORF regions are illustrated in figures 1 and 2.

In some embodiments, the promoters of the present invention are incorporated into vectors that can replicate in a host organism. Such vectors are independently-replicating nucleic acid molecules including, for example, plasmids, phages, and viruses. The vectors can be used to transform eukaryotic cells, for example, plant cells, fungi or other eukaryotic cells. Such transformed plant cells include monocotyledonous and dicotyledonous plant cells.

In some embodiments, the invention is a nucleic acid comprising the promoter region operably linked to a sequence encoding a polypeptide or an RNA molecule. Non-limiting examples of a polypeptide include, a fluorescent protein (See, e.g., Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994), Green fluorescent protein as a marker for gene expression. *Science* 263: 802-805); chloramphenicol acetyl transferase; a protein of pharmaceutical interest; and a protein providing a host plant with pest resistance. A non-limiting example of an RNA molecule that can be expressed from a promoter of the present invention is an anti-sense RNA molecule for modulating the expression levels of an endogenous plant gene.

The invention further contemplates methods of demonstrating promoter activity for putative BRRV promoters. In this method, a putative BRRV promoter is operably linked to a reporter gene, for example a DNA encoding a green fluorescent protein or chloramphenicol acetyltransferase. A transgenic plant or plant cell is then generated by transforming the plant or plant cell with the resulting construct. The plant or plant cell, or a descendant thereof comprising the chimeric construct, is then assayed for the presence of the product of the reporter gene. In preferred embodiments, tissues from developing or mature plants are analyzed for the tissue-specific expression. "Tissue-specific expression" is expression of an RNA transcript or a protein product of translation of a transcript, that is at least five times greater than, more preferably at least ten times greater than, and more preferably at least twenty times greater in one tissue compared to another, when measured as a percentage of total RNA synthesis or total polypeptide synthesis in each tissue. One

example of a promoter region identified from BRRV is an 821 bp sequence (SEQ ID NO: 9).

In some embodiments, the invention provides a plant or plant cell comprising a recombinant sequence derived from BRRV DNA. Preferably, the plant or plant cell comprises a recombinant sequence derived from BRRV DNA comprising a BRRV promoter sequence. More preferably, the plant or plant cell comprises a recombinant sequence comprising a BRRV promoter sequence operably linked to a nucleic acid encoding a polypeptide or an RNA molecule.

In some embodiments, the invention includes recombinant BRRV promoter constructs comprising multiple copies of a promoter sequence. Tandem repeats of a promoter G sequence or of any of the other promoter sequences can also be made and operably linked to a coding region for a desired expression product such as a reporter gene.

Example 1

This example demonstrates isolation, characterization, and sequencing of BRRV DNA.

Blueberry red ringspot virions were isolated from blueberry leaf tissue using the technique of Gillett and Ramsdel (Gillett, J. M., and Ramsdell, D. C. (1984). Detecting the Inclusion forming Blueberry Red Ringspot Virus with ELISA. *Phytopathology* 74: 862). To obtain a sequence of BRRV, viral DNA, obtained from an infected blueberry plant in 1989, was digested with the restriction enzyme Eco RI. Separately, viral DNA obtained from infected blueberry plants at a later date was digested with restriction enzyme Xba I. Fragments digested with either enzyme were ligated into pUC 119 vector digested with either the Eco RI or the Xba I restriction enzyme. Recombinant plasmids were grown and maintained in *E. coli* DH5 alpha cells. Sequences of DNA fragments inserted into the pUC 119 vectors were determined using an ABI automated sequencer and "Big Dye" terminator technology. Once initial sequence was determined, unique primers were designed to "walk" through the sequence of the clone. Sequences obtained from the Xba I and Eco RI digests were then compared, and overlapping sequences were used to determine the relative positions of the fragments in an undigested virus.

Example 2

This example demonstrates promoter activity in a recombinant fragment of BRRV sequence.

To demonstrate promoter activity in a fragment of BRRV sequence, an 821 bp fragment (SEQ ID NO: 9) was identified as containing a TATATAA box, at least one cis-acting transcription element, and a translation start sequence less than 100 base pairs from the TATATAA box. This fragment was operably linked to a cDNA encoding a green fluorescent protein (GFP). The resulting recombinant construct was used as a transgene to transform plants of the species *Arabidopsis thaliana* using standard procedures. Plant tissues comprising the transgene were assayed for fluorescence indicative of the presence of GFP. All tissues examined exhibited fluorescence, indicating that the sequence of the 821 bp fragment provided promoter activity that was able to direct the expression of the GFP cDNA

Example 3

This example demonstrates promoter activity of putative BRRV promoters.

To investigate promoter activity in putative BRRV promoters, a GFP cDNA was operably linked to putative promoters of BRRV identified as above. Included in the analysis are assays of For comparison, the GFP cDNA was also operably linked to a 35 S promoter of Cauliflower Mosaic Virus (CaMV). *Arabidopsis thaliana* plants were transformed, and transgenic plant tissues, in particular apical meristem, root, and leaf tissue were assayed for the presence of GFP. Relative brightness of fluorescence was estimated by eye using plants transformed with a construct comprising the CaMV 35 S promoter operably linked to GFP cDNA as a standard.

The lengths of putative promoter sequences tested for their ability to support expression of GFP cDNA in *Arabidopsis thaliana* tissue are as follows:

Promoter A - 423 nucleotides
Promoter B - 429 nucleotides
Promoter C - 428 nucleotides
Promoter D - 451 nucleotides
Promoter E - 401 nucleotides
Promoter F - 423 nucleotides
Promoter G - 447 nucleotides

The results of testing the promoters are presented in Table 1. For promoter G, both truncated sequence fragments as well as tandem duplications of the

sequence were also tested for their ability to direct GFP expression. Note that one putative promoter, Promoter A, provides the ability to direct tissue-specific expression of the transgene to the roots.

Table 1[a]

Promoter:	Apical Meristem	Root	Leaf
BRRV G	+	N/A[b]	+
BRRV G Short (221 bp)	+	+	(+)[c]
BRRV G Shortest (219 bp)	+	-	-
BRRV G tandem repeat	N/A[b]	+	+
BRRV A	-	++	-
CaMV 35S	+++	+++	+
BRRV E	-	+	+
BRRV F	-	+	+

[a] "+++" indicates tissue strongly fluorescent for GFP. "++" indicates tissue exhibiting GFP fluorescence, but less intense than that of comparable tissue from plants transformed with the CaMV 35 S-GFP construct. "+" indicates tissue exhibiting GFP fluorescence, but less intense than that of comparable tissue exhibiting "++" intensity fluorescence. "(-)?" indicates extremely weak, possibly artificial, fluorescence.

[b] N/A not attempted

[c] Fluorescence detectable but weak.

Example 4

This example demonstrates discovery and demonstration of a minimal promoter derived from the BRRV DNA sequence.

To isolate a minimal promoter from BRRV, portions of the 821 base pair sequence described above were made. One such fragment, containing only 385 base pairs of DNA, was operably linked to the gene encoding green fluorescent protein, and the resulting construct was used to transform *Arabidopsis thaliana* plants as above. Leaf tissue from these plants exhibited fluorescence indicative of

the presence of the green fluorescent protein, thereby demonstrating that a sequence no larger than 385 base pairs comprising promoter G exhibits minimal promoter activity, and can be used to direct expression of heterologous genes in transgenic organisms.

Example 5

This example demonstrates the determination of a consensus sequence for a putative minimal promoter.

To determine a consensus sequence for a putative minimal promoter, a computer was used to align the sequences from each of the 7 putative promoters described above. Using standard sequence analysis algorithms, a consensus sequence was derived (SEQ ID NO: 10). This consensus sequence is notable for its high A-T content (approximately 90%).The aligned consensus sequence is shown in Figure 2 in which consensus bases are shown in capital letters. Dashes represent more than one base as indicated in the possible bases at that position in the seven aligned sequences.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description be interpreted as illustrative and not in a limiting sense.

The inventor contemplates that many variations on the disclosed sequences, such as insertions, deletions, inversions, duplications, and substitutions are contemplated as within the scope of this invention. The inventor also contemplates that the promoters and their variants will exhibit distinct expression patterns in terms of both species specificity, tissue specificity and developmental specificity. The inventor also contemplates that sequences hybridizing to the blueberry red ringspot virus under stringent conditions, particularly the putative promoter sequences, will reveal useful activity, in particular promoter activity for use in directing expression of transgenes. It is contemplated that blueberry red ringspot virus nucleic acid sequences having lengths of about 10, 15, 20, 25, or 50 base pairs can be used for hybridization under stringent conditions to isolate new examples of useful sequences having homology to the virus. Sources of DNA for hybridization can be other viruses, plants, animals, fungi, and prokaryotes.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference

constitutes prior art relevant to patentability. Applicant reserves the right to challenge the accuracy and pertinency of the cited references. As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description be interpreted as illustrative and not in a limiting sense.

WHAT IS CLAIMED IS:

1. A recombinant nucleic acid comprising a sequence having promoter activity in a plant cell and which hybridizes under high stringency conditions to a fragment of a blueberry red ringspot virus nucleic acid which has promoter activity in the plant cell, or the complement thereof.

2. The recombinant nucleic acid of claim 1 wherein the plant cell is a dicotyledonous plant cell.

3. The recombinant nucleic acid of claim 1 wherein the plant cell is a monocotyledonous plant cell.

4. The recombinant nucleic acid of claim 1 wherein the sequence comprises a consensus promoter sequence as set forth in SEQ ID NO: 10.

5. The recombinant nucleic acid of claim 1 comprising BRRV promoter region A as set forth in SEQ ID NO: 2, BRRV promoter region B as set forth in SEQ ID NO: 3, BRRV promoter region C as set forth in SEQ ID NO: 4, BRRV promoter region D as set forth in SEQ ID NO: 5, BRRV promoter region E as set forth in SEQ ID NO: 6, BRRV promoter region F as set forth in SEQ ID NO: 7 or BRRV promoter region G as set forth in SEQ ID NO: 8.

6. A nucleic acid probe or primer of at least 10 consecutive nucleotides of the sequence as set forth in SEQ ID NO: 1 or the complement thereof, wherein said probe or primer specifically hybridizes under stringent conditions to a blueberry red ringspot virus nucleic acid sequence.

7. The nucleic acid probe or primer of claim 6 which specifically hybridizes under stringent conditions to a promoter region comprising BRRV promoter region A as set forth in SEQ ID NO: 2, BRRV promoter region B as set forth in SEQ ID NO: 3, BRRV promoter region C as set forth in SEQ ID NO: 4, BRRV promoter region D as set forth in SEQ ID NO: 5, BRRV promoter region E as set forth in SEQ ID NO: 6, BRRV promoter region F as set forth in SEQ ID NO: 7, BRRV promoter region G as set forth in SEQ ID NO: 8, or a BRRV promoter region as set forth in SEQ ID NO: 9.

8. A recombinant nucleic acid comprising a promoter from the blueberry red ringspot virus operably linked to a DNA sequence encoding a polypeptide or an RNA.

9. The recombinant nucleic acid of claim 10 wherein the promoter comprises BRRV promoter region A as set forth in SEQ ID NO: 2, BRRV promoter region B set forth in SEQ ID NO: 3, BRRV promoter region C set forth in SEQ ID NO: 4, BRRV promoter region D set forth in SEQ ID NO: 5, BRRV promoter region E set forth in

SEQ ID NO: 6, BRRV promoter region F set forth in SEQ ID NO: 7, BRRV promoter region G as set forth in SEQ ID NO: 8, or a BRRV promoter region as set forth in SEQ ID NO: 9.

10. The recombinant nucleic acid of claim 9 wherein the promoter region is operably linked to a gene encoding a polypeptide or an RNA.

11. A transgenic plant cell comprising the recombinant nucleic acid of claim 10.

12. A transgenic dicotyledonous plant comprising the plant cell of claim 11.

13. A transgenic monocotyledonous plant comprising the plant cell of claim 11.

14. The transgenic plant of claim 11, wherein the plant is of the species *Arabidopsis thaliana*.

15. A method for transforming a plant cell comprising transforming a plant cell with a recombinant DNA construct comprising a blueberry red ringspot virus promoter and a DNA sequence which encodes a polypeptide or an RNA; wherein the promoter regulates the transcription of the DNA sequence.

16. A method for diagnosing the presence of blueberry red ringspot virus in a host plant comprising (a) incubating a nucleic acid sample obtained from a plant suspected of containing the virus with a nucleic acid probe of claim 6 under conditions in which the probe can hybridize with any blueberry red ringspot virus nucleic acid present in the sample and (b) detecting the presence of any hybridization complex formed.

17. A method for diagnosing the presence of blueberry red ringspot virus in a host plant comprising (a) providing two oligonucleotides which are primers for a polymerase chain reaction method and which flank a target sequence which lies within a blueberry red ringspot virus nucleic acid as shown in Figure 2 or the complement thereof; (b) incubating the oligonucleotides with a nucleic acid sample obtained from a plant suspected of containing the virus; (c) amplifying the target DNA sequence if it is present in the nucleic acid sample by the polymerase chain reaction method and (d) detecting the presence of the any amplified target DNA sequence.

Promoter Designation	TATATAA Box Location	Nucleotide's Separating TATATAA Box From AUG
A	7,051 bp	27
B	7,739 bp	56
C	8,877 bp	19
D	1,298 bp	22
E	1,795 bp	72
F	2,407 bp	41
G	5,314 bp	18

FIGURE 1

```
Plurality: 2.00  Threshold: 1  AveWeight 1.00  AveMatch 1.00  AvMisMatch 0.00

 Consensus Sequence

 Symbol comparison table: pileupdna.cmp CompCheck: 6876

              GapWeight: 5
        GapLengthWeight: 1

PileUp  MSF: 206  Type: N  December 6, 2000 12:09  Check: 1484 ..

 Name: putpromotera     Len:   206  Check: 8733  Weight:  1.00
 Name: putpromoterd     Len:   206  Check: 2099  Weight:  1.00
 Name: putpromotere     Len:   206  Check: 6802  Weight:  1.00
 Name: putpromoterf     Len:   206  Check: 5015  Weight:  1.00
 Name: putpromoterg     Len:   206  Check: 6944  Weight:  1.00
 Name: putpromoterb     Len:   206  Check: 8395  Weight:  1.00
 Name: putpromoterc     Len:   206  Check: 3416  Weight:  1.00


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putpromoterd ---------- ---------- ---------- ---------- -------ata
putpromotere acgatgcgaa gatgaaggaa tcatcttatc acagccaaaa gcaaaaatag
putpromoterf ---------- ---------- ---------- ---------- ---------a
putpromoterg ---------- ---------- ---------- ---------- ----------
putpromoterb ---------- ---------- ---------- ---------- ---------g
putpromoterc ---------- ---------- ---------- ---------- ---------a
   Consensus ---------- ---------- ---------- ---------- ---------A

              51                                                 100
putpromotera -acactatcc gagggaatgc ccaacctaca aaagaaatga tcatcgataa
putpromoterd gaaaaagtcc acatagtgcg ccagcattct atgtagaaaa tcattcagaa
putpromotere cacataaaga aattgatttc tttggattca tatttcagaa ggagaaataa
putpromoterf tttactatca aaacctttta ttttaaaaac agattcaaaa tatgttacag
putpromoterg ------atag aataatttct aaaaataaga aagaacaagt tctaggaata
putpromoterb gtccagtaac aggacgatta gaagatggaa tattaaatct agattgttta
putpromoterc taaagtagcc cttatgatta caatattaat agaaaaaagg aaattccttg
   Consensus -ACA-TATCC AATA-GTTTA CAAGA-TA-A AAATA-AA-A TCATTGATAA

              101                                                150
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putpromoterb actaatggag aagaattatt aaaacaatgg acagcaaaac aatcattatc
putpromoterc tacccactat atatcaattt gattcaggag taccaatgat tataggaaat
   Consensus -ATAAAAGAG ATATAA-ATT AAAACAATTA A-ATTAAAA- CAT-TGAATT

              151                                                200
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putpromoterd aagccaccat tggaacacct aaacattacc cagagcagat tatataa---
putpromotere cagaatcgaa agcaattaca aagatttctt ggaaatttaa attatataa-
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putpromoterg tcgccatacc attagtaact aagaatttaa gccaatctat aggaatatta
putpromoterb aacacaaatt gatgctacca tccgagacat ggatgctgag aatatataa-
putpromoterc aactttttaa gattatatta cccatttgt caatatctat cttatataa-
   Consensus AAGCC--AAA T-TAATAACA AA-AATTTAT -AAA-TT-AT --TATATAAA

            . 201
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putpromoterd ------
putpromotere ------
putpromoterf ------
putpromoterg tataaa
putpromoterb ------
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FIGURE 2

SEQUENCE LISTING

<110> Michigan State University
Allison, Richard

<120> Blueberry Red Ringspot Virus, Sequences, Promoters, and Uses Thereof

<130> 6550-000064/POA

<150> 60/318,050
<151> 2001-09-07

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/28260

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; C12N 5/04, 5/10, 15/00, 15/70, 15/82, 15/87; C07H 21/04; A01H 5/00

US CL. : 435/6, 320.1, 419, 468; 536/24.1, 24.3, 24.33, 24.1; 800/278, 295, 298

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 320.1, 419, 468; 536/24.1, 24.3, 24.33, 24.1; 800/278, 295, 298

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, Agricola CAplus, Biosis, EMBL, Geneseq, EST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HEPP et al. Bluebery Red Ringspot Virus Detection in Crude Sap of Highbush Blueberry Plants. Plant Disease, June 1987, Vol. 71, No. 6, pages 536-538. see whole document.	16, 17
X	MAITI et al. Gene Expression Regulated By Gene VI of Caulimovirus: Transactivation of	1-5, 8-15
---	Downstream Genes of Transcripts by Gene VI of Peanut Chlorotic Streak Virus in	----------
Y	Transgenic Tobacco. Virus Resarch, 1998, vol. 57, pages 113-124, especially pages 117-121.	6, 7, 16, 17
X	MAITI et al. Isolation and Expression Analysis of Peanut Chorotic Streak Caulimovirus	1-5, 8-15
---	(PCISV) Full-Length Transcript (FL2) Promoter in Transgenic Plants. Biochem. Biophys.	----------
Y	Res. Comm. 1998. Vol. 244, pages 440-444, especially pages 441-443.	6, 7, 16, 17
T, A	GLASSHEN, et al. Cloning, Sequencing, and Promoter Identification of Blueberry Red Ringspot Virus, a Member of the Family Caulimoviridae with Similarities to the "Soybean Chlorotic Mottle-Like" Genus. Arch. Virol. 2002. Vol. 147, pages 2169-2185, see whole document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 December 2002 (02.12.2002)	18 DEC 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Ashwin Mehta Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/28260

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/US02/28260

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-5 and 8-15, drawn to a first product, any recombinant nucleic acid comprising any sequence having promoter activity in a plant cell and which hybridizes under high stringency conditions to any fragment of blueberry red ringspot virus nucleic acid which has promoter activity in the plant cell; a recombinant nucleic acid comprising any promoter from blueberry red ringpot virus (BRRV) operably linked to any DNA sequence encoding a polypeptide or RNA; any transgenic plant cell or plant comprising said recombinant nucleic acid; and a first method, comprising transforming any plant cell with any recombinant DNA construct comprising any BRRV promoter and any DNA sequence encoding any polypeptide or RNA.

Group II, claim(s) 6, 7, 16, and 17, drawn to a second product, a nucleic acid primer or probe, and a second method, for diagnosing the presence of BRRV in a host plant, comprising incubating a nucleic acid sample from a plant from a plant suspected of containing the virus with said nucleic acid probe, or providing two oligonucleotides which are primers and amplifying a target DNA sequence.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the promoters of Group I are not shared with the products or methods of Group II. The method of using the first product of Group I is not shared with the second method of Group II. The groups do not share any special technical features.